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Research Article

The Effects of Formulation Variables on the Stability of Freeze-Dried Human Growth Hormone[1]

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Formulation often has a dramatic effect on degradation of proteins during the freeze-drying process as well as impacting on the "shelf-life" stability of the freeze-dried product. This research presents the results of a formulation optimization study of the "in-process" and shelf-life stability of freeze-dried human growth hormone (hGH). Chemical decomposition via methionine oxidation and deamidation of asparagine residues as well as irreversible aggregation were characterized by HPLC assay methodology. In-process degradation and stability of low moisture freeze-dried solids were studied at 25 and 40°C in a nominal nitrogen headspace (≈0.5% O_2). Formulation variables included pH, level of salts, and the nature of the lyoprotectant. Studies of the effect of shear on aggregation in solutions indicated that shear comparable to that experienced during filtration does not induce aggregation. Irreversible changes in hGH during the freeze-drying process were minimal, but chemical decomposition via methionine oxidation and asparagine deamidation and aggregation did occur on storage of the freeze-dried solid. Decomposition via methionine oxidation was significant. A combination of mannitol and glycine, where the glycine remains amorphous, provided the greatest protection against decomposition and aggregation. It is postulated that an excipient system that remains at least partially amorphous is necessary for stabilization. However, the observation that dextran 40 formulations showed poor stability toward aggregation demonstrates that an amorphous excipient system is not a sufficient condition for stability. Stability of the solid was optimal when produced from solutions in the pH range, 7–7.5, with severe aggregation being observed at high pH. The level of sodium phosphate buffer affected stability of the solid, although this relationship was complex. Freeze-drying in the presence of NaCl produced severe aggregation and precipitation during the freeze-drying process as well as acceleration of oxidation and/or deamidation.

KEY WORDS: freeze-drying; stability of proteins; lyoprotectants; protein formulation; human growth hormone (hGH).

INTRODUCTION

A number of new therapeutic proteins have been marketed in recent years (1). A successful product must survive processing and storage over the claimed shelf life without excessive loss of potency or excessive increase in the level of decomposition products. Proteins generally present stability problems which are more complex than those encountered with small molecules (1). Not only does the diversity of primary structure create the potential for numerous chemical decomposition pathways, but the possibility of conformational changes adversely affecting potency introduces another dimension to stability considerations. Many protein products are freeze-dried to provide adequate shelf-life stability (1). While a freeze-dried solid is generally much more stable than the corresponding aqueous solution, some proteins are inactivated during the freeze-drying process; the extent of inactivation is extremely sensitive to formulation (2–5). In addition, degradation and loss of activity may occur during storage, the extent of the stability problem being sensitive to both formulation variables and the level of residual water in the nominally dry solid (5–7). While mechanistic interpretations have been offered for the effect of residual water (6–8) and "lyoprotectants" such as sugars (9–11) on the stability of freeze-dried proteins, a considerable degree of empiricism exists in the formulation of a new protein product. Selection of a formulation is often dominated by the perceived need to select excipients which are historically well accepted for use in parenteral therapy.

Human growth hormone (hGH) is a 22-kD protein which is approved for use in treating hypopituitary dwarfism via im injection. In both aqueous solution and the solid state, hGH undergoes chemical decomposition via oxidation and deamidation; the major oxidation product is the Met-14 sulfoxide, and the major deamidation site is Asn-149 (12). Human growth hormone also forms dimers and higher molecular weight aggregates where, at least for the species studied by Becker and co-workers (13), the dimer appears to be a noncovalent aggregate identical in primary structure to native hGH.

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This research is a study of the effect of formulation on the "in-process" and storage stability of freeze-dried hGH. The objective is formulation optimization. Effects of residual water content and oxygen in the vial headspace are addressed elsewhere (14). In-process decomposition and stability of the freeze-dried solids at 25 and 40°C were monitored via HPLC assays for chemical decomposition and aggregation. The formulation variables studied include pH, level of salts, and the nature of the potential "lyoprotectant" excipient: mannitol, glycine, dextran 40, and lactose.

EXPERIMENTAL

Materials

The hGH was obtained as raw material (Eli Lilly & Co.). Except when otherwise noted, all stability studies were conducted using the same raw material batch of hGH (lot HG6), and all hGH solutions were buffered to pH 7.4 with 0.277 mg $Na_2HPO_4 \cdot 7H_2O$ per mg hGH, with final pH adjustment with NaOH or H_3PO_4. Dextran 40 was obtained from Pharmacia, and all other chemicals were either USP or reagent grade. The water used was water for injection (Eli Lilly & Co.). The vials were 5-ml tubing vials (type I glass) with a 13-mm neck (Wheaton Co.). The stoppers were obtained from the West Co. (West 1816 rubber). All chemicals and container components were used as received without further purification or treatment except for washing the vials.

Assays

Residual moisture was determined by coulometric Karl Fischer assay (Aquastar, EM Science). Accuracy of the Karl Fischer assays for hGH systems was verified by comparing Karl Fischer results with "loss on drying" techniques (TGA at moderate water content and zero water content by vacuum drying at $\approx 10^{-6}$ mm Hg and 25°C). Reported moisture contents normally represent means of at least three independent replicates (i.e., different vials) and are estimated to be accurate within $\approx \pm 0.2\%$ water.

Decomposition of hGH via methionine oxidation and deamidation was measured by reverse-phase HPLC (UV detection at 220 nm) using a Vydac C4 column with a mobile phase of 29% *n*-propanol and 71% 0.05 *M* Tris buffer at pH 7.5 (15). In this system, the major oxidation product at Met-14 and the major deamidation product at Asn-149 have essentially the same retention time. For selected samples, deamidated hGH was separated from native hGH and oxidized hGH by anion-exchange chromatography (UV detection at 280 nm) using a Mono-Q column (15). The mobile phase was prepared using 0.05 *M* Tris buffer at pH 8 and acetonitrile (70/30, mobile phase A). The B mobile phase was prepared from the A mobile phase by the addition of 0.3 *M* NaCl. The separation was performed using a linear gradient from 0 to 100% B over 25 min (15). Chemical purity as defined by the reverse-phase HPLC assay, denoted "reverse-phase purity," was calculated from the area of the "main peak" (undecomposed hGH) and the sum of peak areas for all related substances. Soluble aggregates (dimer and higher order) were determined by size exclusion HPLC (UV detection at 214 nm) under nondenaturing conditions (16), using a Dupont GF250 column. The mobile phase was 0.025 *M* NH_4HCO_3. The percentage monomer was calculated from the peak areas of monomer, dimer, and higher molecular weight aggregates. The assay methodology does not distinguish between covalent and noncovalent aggregates. All HPLC assays assume that the extinction coefficient of the degradation product is the same as for the native protein. Reported assay results are normally the result of assays on duplicate samples (i.e., two different sample vials). Based on agreement between duplicates assayed on the same day and reproducibility of a reference standard stored at −20°C over the duration of the studies, the usual standard errors in the HPLC assays are estimated to be ±0.4% for initials and ±0.6% for samples stored at 25 and 40°C.

Measurement of Physical Properties

Collapse temperatures were determined using the freeze-drying microscope technique (17). DSC (Perkin-Elmer DSC-7) scans were employed to search for glass transition temperatures (18) in the solids over the temperature region of interest, using a scan rate of 10°C/min and a total solid sample of about 10 mg in sealed pans. To ensure that "start-up" artifacts were dissipated before a temperature of 0°C was reached, all scans were started at −20°C. A scan up to 60°C was made on a "fresh" sample, the same sample was cooled to −20°C, and another scan was made up to a maximum temperature of 180°C.

Freeze-Drying Procedures

Vials were filled with 1 ml of 2 mg/ml hGH solution and loaded on a freeze-dryer (Virtis 25-SRC-X) shelf at ≈5°C. After thermal equilibration, the shelf temperature was lowered to −40°C. The product supercooled to ≈ −12°C, ice nucleated, and freezing was complete within ≈15 min. After cooling to ≈ −40°C, primary drying (or ice sublimation) was initiated. The temperature of the frozen product was maintained between −30 and −35°C for the duration of primary drying (≈5 hr). The samples then remained at the shelf temperature (−10°C) until the next morning (≈10 hr), after which the shelf temperature was increased to complete secondary drying. This stage of secondary drying was carried out at a product temperature of 30°C for 4 hr, after which the drying chamber was vented with dry nitrogen, and the vials were sealed by stoppering in the freeze-dryer. All samples freeze-dried with full retention of structure (i.e., no collapse, melt, or glass transition evident from visual or microscopic examination).

RESULTS AND DISCUSSION

Properties of hGH Formulations

The composition and physical properties of the hGH formulations are summarized in Table I. Note that the water content increases upon storage. Based on other studies in our laboratories (unpublished), this increase in water content arises from absorption of water from the stopper, not transmission of water through the stopper, and the data given for "aged" samples are roughly the level of water at equilibrium for both 25 and 40°C storage. The residual water content

Table I. Properties of hGH Formulations[a]

Excipient code	Excipient weight ratio hGH:glycine:mannitol:dextran	% water		Collapse temperature[c]		Crystallinity (X-ray diffraction)
		Initial	Aged[b]	Fast freeze	Slow freeze	
A	1:0:0:0	1.4	5.	> −5°C	> −5°C	Amorphous
B	1:1:0:0	0.3	2.4	> −5°C	> −5°C	Glycine crystals
C	1:0:5:0	0.6	1.3	−22°C	> −5°C	Mannitol crystals
D	1:1:5:0	0.3	1.2	−24°C	> −5°C	Mannitol crystals
DEX	1:0:0:6	0.8	—			Amorphous

[a] Each sample contained 0.091 mmol hGH in a 5-ml vial. Residual headspace oxygen averaged 0.4% (σ = 0.2%).
[b] Aged for between 4 and 5 months at 25°C. Note that 1% residual water in pure hGH is ≈12 mol H_2O per mol of hGH.
[c] Collapse temperatures were measured for the excipient systems buffered to pH 7.4 with 0.227 mg $Na_2HPO_4 \cdot 7H_2O$ buffer per mg hGH (1.69 m*M* buffer).

varies with formulation largely because, while the percentage water is based on the total sample mass, the crystalline components do not retain significant amounts of water during freeze-drying, nor do crystalline components sorb water from the stopper during storage. Collapse temperature data show a sensitivity to the thermal history of the sample, denoted "fast freeze" and "slow freeze." A normal freezing procedure in a freeze-drying microscope experiment is very rapid relative to freezing in a vial and is denoted fast freeze. A freeze-drying microscope approximation to the thermal history of a sample freezing in a vial is obtained by first freezing rapidly, increasing the temperature to ≈ −15°C, holding at this temperature for several minutes, and then decreasing the temperature to less than −30°C to start the collapse temperature measurement. This procedure is denoted slow freeze. It appears that mannitol does not crystallize during the fast-freeze procedure, thereby giving a low collapse temperature for systems C and D. However, during the slow-freeze procedure, as well as freeze-drying in a vial, most of the mannitol does crystallize. Consistent with the statement that microscope slow freeze is a good approximation to freezing in a vial, formulation D can be freeze-dried in a vial at a product temperature of at least −15°C without collapse. While glycine does crystallize in system B, where glycine is the only lyoprotectant, the glycine:mannitol combination used in system D prevents crystallization of glycine, although most of the mannitol has crystallized.

Amorphous freeze-dried pharmaceuticals generally exhibit glass transition temperatures. Above the glass transition temperature, high molecular mobility allows greater reactivity; below the glass transition temperature, mobility and therefore reactivity are greatly restricted (8,19,20). Therefore, a sharp increase in reactivity is expected at those storage temperatures which are above the glass transition temperature. However, no evidence for glass transitions in the 0–60°C temperature range was revealed by DSC for any of the hGH formulations. A broad endotherm with an onset at ≈40°C and a peak at ≈50°C was observed for each formulation. The energy of the transition was ≈11 mJ for formulation E and ≈6 mJ for all other formulations. Release of water into the "headspace" of the sealed DSC pan is one possible cause for this endotherm. Scans up to 180°C display the sharp increase in baseline characteristic of a glass transition for the dextran formulation (≈85°C). With mannitol-containing formulations, a strong endotherm is observed at ≈150°C (peak), which is probably due to melt of mannitol (*pure* mannitol melting point = 165°C, CRC handbook). Possible glass transitions for formulations B, C, and D are observed in the range 100–130°C. While these high-temperature transitions have no direct relevance to stability at 40°C and below, the endotherm with an onset near 40°C may, in principle, be relevant to stability of the solid at 40°C. However, neither the peak temperature nor the heat of transition shows any obvious correlation with the stability data presented later in this report. The only DSC observation of obvious relevance to solid-state stability is that no glass transitions were observed in the temperature range, $t \leq 40$°C.

Making the plausible, though not rigorous, assumption that only a single amorphous phase exists, we may tentatively conclude that the protein phase in system D consists of a 1:1 weight ratio of hGH:glycine with, at most, a small amount of dissolved mannitol and phosphate buffer. Note that the X-ray evidence for crystalline mannitol does not preclude the possibility of some of the mannitol distributing into the amorphous phase. Due to limited sensitivity of the X-ray method, crystalline buffer could go undetected for those systems with crystalline components. However, since microscopic examination (i.e., birefringence) and X-ray data for system A indicate that most of the buffer remains amorphous, it seems likely that the buffer is mostly amorphous in the other systems as well. While the exact composition of the hGH phase is somewhat uncertain, it is obvious that systems D and DEX have the greatest potential for producing some effect on the stability of hGH. In both these systems, the "lyoprotectant" is at least partially amorphous and therefore has the greatest probability of being distributed in the protein phase to interact molecularly with the hGH and/or physically separate or "dilute" the hGH molecules. This dilution effect would be expected to decrease the formation of aggregates during processing and storage. If dilution is the only effect, the DEX system would be more effective than system D, since with DEX there exists a 6:1 dilution rather than the nominal 1:1 dilution with system D.

In-Process Chemical Decomposition and Aggregation

Shear-Induced Aggregation in Mechanically Agitated Solutions. The tendency of hGH solutions to aggregate and ultimately precipitate when shaken is well known. Often the effect is attributed to shear forces, although loss of protein

potency is expected only at very high values of the "average shear rate:residence time" product, $S \cdot t > 10^5$, where S is the shear rate and t is the time of exposure to this shear rate (21). In shaking, denaturation at the air–water interface is possible, and if rubber or silicone oil is present, denaturation at the aqueous:hydrophobic interface may be the main cause of aggregation and precipitation. To determine whether or not there is a pure shear-induced aggregation in hGH solutions, hGH solutions were pumped via a syringe through capillary tubes of various lengths at selected flow rates. For flow through tubes, the average shear rate:residence time product is determined (21) by the length, L, and radius, r, of the tube: $S \cdot t = (8/3)(L/r)$. The end of the capillary tube was positioned below the surface of an hGH solution in a small beaker. This procedure allows the sheared solution to be collected without the introduction of additional air–water interface. The capillary tubing diameter and length were chosen to match shear rate · residence time products experienced by solutions being filtered through 0.22-μm filters, $S \cdot t \approx 10^3$–10^4. Intuitively, the "$S \cdot t$" product would be much smaller for the mechanical agitation inherent in a shaking experiment. Even at the highest shear rates and residence times studied, the sheared solutions were clear, were free of particulates, and showed no significant increase in soluble aggregates (HPLC) relative to the solution before shear (Table II). Thus, we conclude that hGH solutions are not subject to *shear-induced* aggregation under normal processing or handling conditions. The aggregation and precipitation observed on shaking (22) are most likely a result of denaturation at an aqueous:hydrophobic interface, followed by aggregation and ultimate precipitation.

Degradation on Freeze-Drying. Representative data given in Fig. 1 demonstrate that hGH suffers little degradation during freeze-drying. Relative to samples freeze-dried without a lyoprotectant (formulation A), the glycine: mannitol excipient system (formulation D) does appear to retard formation of aggregates, although the difference between formulation A and formulation D is small (≈0.5%). A higher pH (pH 8), a small loss (≈2%) of reverse-phase purity does occur on processing. These observations are representative of all samples studied except those formulations containing NaCl. Extensive aggregation and precipitation (i.e., visual observation of "haze" or turbidity) were observed on freeze-drying in the presence of NaCl. We conclude that hGH of high purity can be freeze-dried without significant chemical decomposition or aggregation, even without the use of a lyoprotectant.

Table II. Results of Hydrodynamic Shear on Aggregation of hGH Solutions[a]

Analytical parameter	Before shearing	After shearing
Clarity (visual)	Clear	Clear
Fibrils or ppt (visual)	None	None
SEC HPLC		
% monomer	98.3	98.0
% dimer	1.3	1.6
% trimer and higher aggregates	0.4	0.4

[a] Results for flow of 2.5 ml/min through a 0.5-mm-diameter glass capillary tube of 50-cm length. $S \cdot t = 8 \cdot 10^3$. The hGH (2.6 mg/ml) was from formulation D.

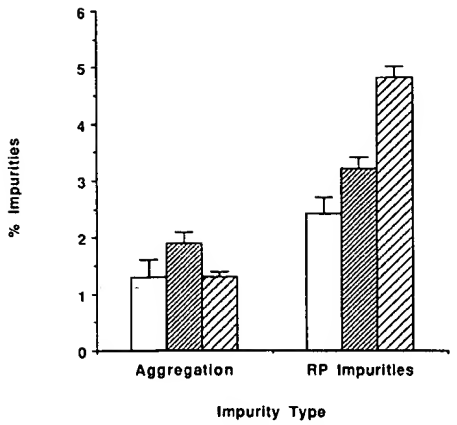

Fig. 1. Changes in hGH during freeze-drying. Aggregation (pH range, 6–8): unshaded, starting solution; dark shading, excipient system A; light shading, excipient system D. Reverse-phase (RP) impurities (excipient systems A and D): unshaded, starting solution; dark shading, pH range of 6–7.4; light shading, pH 8. Systems of pH 7.4 are phosphate buffered (0.227 mg $Na_2HPO_4 \cdot 7H_2O$/mg hGH), while solutions of other pH's were adjusted to the appropriate pH by titration with H_3PO_4 or NaOH.

Kinetics of Chemical Decomposition and Aggregation in the Solid State

Decomposition kinetics as measured by reverse-phase HPLC (methionine oxidation and deamidation) are generally consistent with first-order kinetics (Fig. 2). That is, deviations from linearity on a log (purity) vs time plot are random and probably accounted for by assay variation and variation in water content and oxygen (headspace) between nominally identical samples. Thus, the stability comparisons presented later are generally based on apparent first-order rate constants. While apparent first-order kinetics could be interpreted as evidence for a unimolecular rate-determining decomposition step, this conclusion is highly speculative given the multitude of potentially interdependent hGH decomposition pathways, as well as the general complexity of decomposition kinetics in solids.

In general, the observed aggregation kinetics are not first order; the aggregation rate decreases rapidly with increasing time, with little increase in aggregation during the last half of the time interval studied (Fig. 3). Some systems, however, are consistent with first-order kinetics (i.e., formulation D at 25°C). We assume that the aggregates exist in the solid and are not created from conformationally altered monomer at the instant of reconstitution. While plausible, this assumption is not rigorous, as our methodology for detecting aggregation obviously depends on dissolving the hGH before assay. The observation of aggregation developing in samples stored in the solid state at temperatures well below their glass transition temperatures is surprising. Given the limited molecular mobility in glassy solids (19), it is difficult to understand how a molecule as large as hGH could undergo the level of motion (rotational and translational) presumably required to participate in a bimolecular protein–

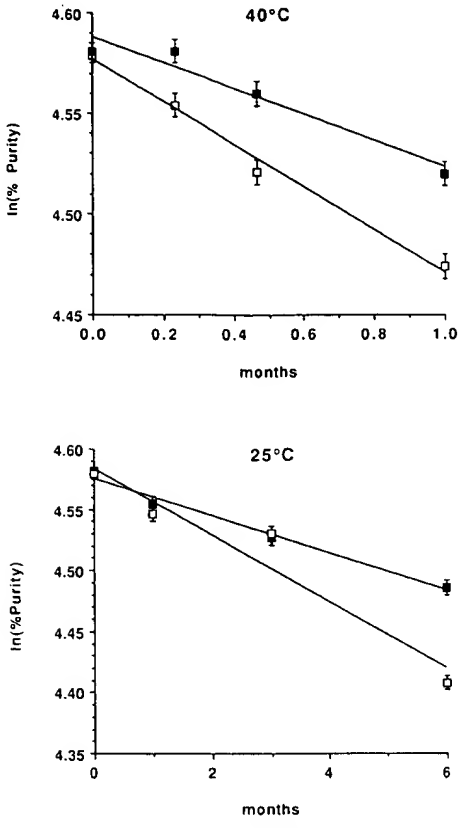

Fig. 2. Representative kinetics for loss of reverse-phase purity of freeze-dried hGH. All samples are buffered with sodium phosphate to pH 7.4 (0.227 mg $Na_2HPO_4 \cdot 7H_2O$/mg hGH). Open squares, formulation A; filled squares, formulation D.

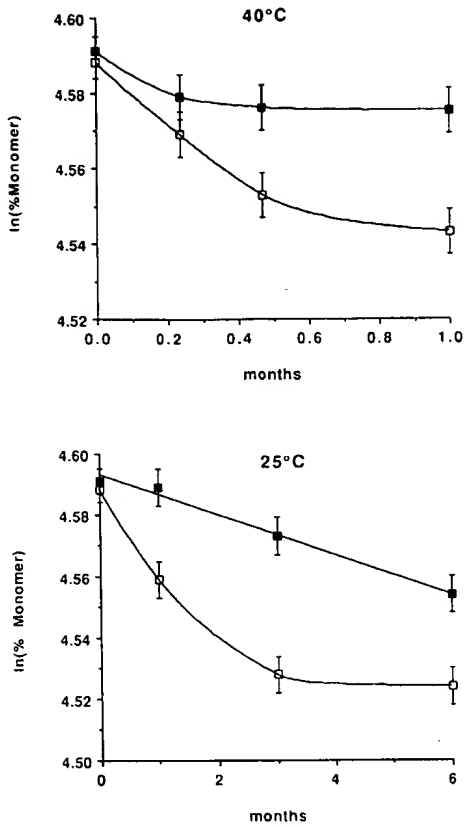

Fig. 3. Representative kinetics for soluble aggregate formation in freeze-dried hGH. Open squares, formulation A; filled squares, formulation D.

protein reaction in the glassy state. However, it does appear that even limited motion is sufficient to allow aggregation of favorably oriented "nearest neighbors." In the amorphous state, the relative orientation of any two molecules is not fixed by "structure" but, rather, is somewhat random. Aggregation of two hGH neighbors will be more or less probable depending on their relative orientation or configuration. This "model" would predict rapid aggregation initially as favorable configurations react but a slowing rate of aggregation as the favorable configurations are depopulated. The observation of apparent first-order aggregation kinetics requires a different interpretation. One could speculate that, under some conditions, the rate of aggregation is limited by the rate of formation of a conformationally altered monomer as an intermediate (22). This type of mechanism would lead to first-order kinetics. Because of the complexity of aggregation kinetics, no attempt to calculate rate constants has been made, and stability comparisons are made by comparing aggregation under fixed conditions of temperature and time.

Reaction Products

Selected samples were assayed both by reverse-phase HPLC and by ion-exchange HPLC. Nominally, the difference between the reverse-phase assay and the ion-exchange assay gives the reaction products which have *only* been oxidized. Reaction products which are both oxidized and deamidated would be measured as deamidated product. The assumptions implicit in this interpretational procedure are that (a) all significant deamidation products are equally measured by both assay methods and (b) reaction products which are neither deamidations nor methionine oxidations are insignificant. These assumptions, while plausible (15), are not rigorous. Results for hGH formulations stored at 25°C in a nominal nitrogen headspace are shown in Fig. 4. The residual oxygen (i.e., the numbers above the bars) is slightly larger for formulation A. Formulation A also shows evidence for significantly more oxidation than the other formulations. It is surprising that even with very low residual oxygen in the vial headspace, decomposition via oxidation is at least as great as decomposition via deamidation. Note that even at 0.4% O_2, the molar ratio of oxygen to hGH is about 10, so from a stoichiometric viewpoint, the supply of oxygen is more than sufficient. However, to react with a hGH molecule in the interior of the amorphous phase, the oxygen must dissolve in the amorphous phase and diffuse to the relevant methionine residue. It is surprising that significant oxygen dissolves at such low partial pressures of oxygen.

For the hGH systems described in Table I, we found no evidence for chemical decomposition other than oxidation or deamidation. However, as an example of a pharmaceutically

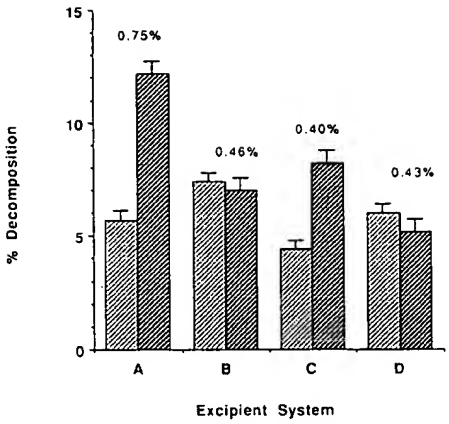

Fig. 4. Comparison of decomposition measured via reverse-phase HPLC with decomposition measured via anion-exchange HPLC for freeze-dried samples stored 6 months at 25°C. The numbers over the bars indicate the residual headspace oxygen in the vials. Light shading, total decomposition via anion-exchange HPLC (nominally, deamidation products); dark shading, difference between reverse-phase and anion-exchange measures of decomposition (nominally, products only oxidized).

acceptable excipient system which would remain totally amorphous, freeze-dried hGH:lactose systems were briefly investigated. The lactose formulations were not pursued because of an apparent additional decomposition mode. HPLC chromatograms (Fig. 5) showed a definite "shoulder" on the main peak in aged samples that was not observed either in the freshly freeze-dried lactose formulation or in any of the other formulations studied. This shoulder was prominent after only 1 month of storage at 25°C and was not sensitive in magnitude or retention time to the presence of oxygen in the vial headspace [i.e., curve $A(N_2)$ vs curve $B(O_2)$]. This shoulder is clearly not present in a mannitol formulation (curve C) and is well removed from the major deamidation peak (curve D). The major oxidation product (not shown) has a retention time essentially the same as the major deamidation product. We postulate that the shoulder represents an adduct of lactose and hGH, either the glycosylamine or the corresponding Schiff base (7).

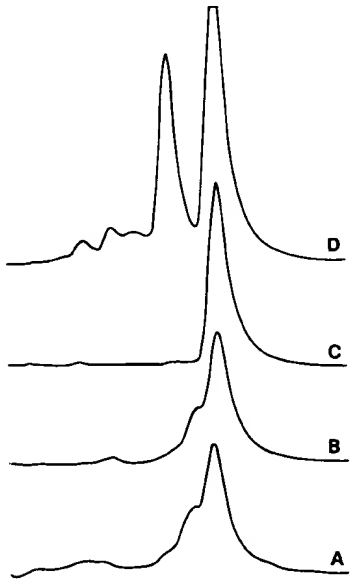

Fig. 5. Reverse-phase HPLC chromatograms of aged freeze-dried hGH formulated with lactose. (A) 1:1 weight ratio of hGH:lactose stored 1 month at 25°C in nitrogen headspace; (B) 1:1 hGH:lactose stored 1 month at 25°C in 90% oxygen headspace; (C) 1:1 hGH:mannitol stored 1 month at 25°C in 90% oxygen headspace; (D) deamidation resolution standard showing peaks due to deamidation.

Formulation Effects

Excipient Systems The effect of "lyoprotectant" excipient system on chemical stability (reverse-phase decomposition) and aggregation of freeze-dried hGH is summarized by Figs. 6 and 7. The error bars represent estimates of the standard error for the stability parameter reported. Aggregation at 3 weeks (40°C) is actually the mean of the 2-week and 4-week data, while aggregation at 2 months (25°C) is the mean of the 1-month and 3-month data. Combining raw data in this fashion reduces the impact of assay error. Relative to excipient system A (no lyoprotectant), glycine (system B), mannitol (system C), and the glycine:mannitol combination

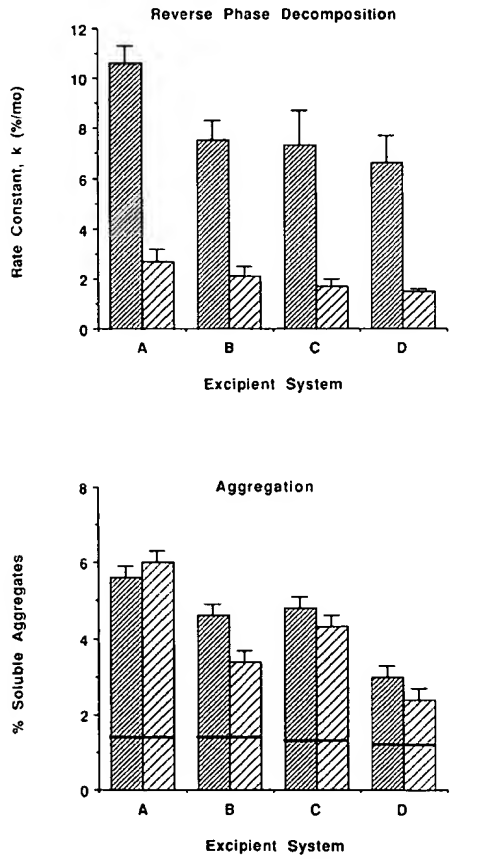

Fig. 6. The effect of excipients on the stability of freeze-dried hGH. The solid horizontal lines in the bottom graph denote the initial level of aggregates in the freeze-dried samples. Reverse-phase decomposition: dark shading, 40°C; light shading, 25°C. Aggregation: dark shading, 3 weeks at 40°C; light shading, 2 months at 25°C.

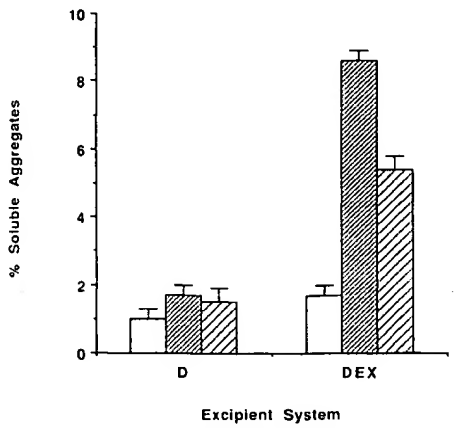

Fig. 7. Comparison of hGH aggregation in the glycine:mannitol formulation (D) with aggregation in the dextran formulation (DEX). Samples for these studies were prepared from raw material lot GR7. No shading, initial solid; dark shading, solid stored for 3 weeks at 40°C; light shading, solid stored for 1 month at 25°C.

(system D) appear slightly more stable both in chemical stability and in resistance to aggregation. Aggregation at both 25 and 40°C decreases in the order A > C > B > D, although the differences between adjacent formulations in this sequence are small. A different hGH raw material batch (data not shown) also resulted in formulation D being more stable toward both chemical decomposition and aggregation than the other formulations. A recent summary also stated that a glycine:mannitol combination was particularly effective in preventing aggregation in solid hGH (24).

Greater stability of formulation D is consistent with the concept that stability enhancement depends on the excipient system being at least partially amorphous to allow molecular interaction with the protein and/or to act as a "sink" for residual water. In formulation D, the glycine is amorphous. However, in the dextran 40 system, the excipient is totally amorphous. If an amorphous excipient were *both* a *necessary* and a *sufficient* condition for stability enhancement, one would expect significantly less aggregation in the dextran 40 formulation. However, quite the opposite effect was observed (Fig. 7). Not only is the level of aggregation immediately after freeze-drying slightly higher in the dextran system than in formulation D, but development of aggregation during storage is much greater for the dextran formulation. Aggregation at 40°C is much greater in the dextran system (Fig. 7) than in formulation A, which contains no lyoprotectant (Fig. 6), even though the raw material lot used for the studies in Fig. 7 is evidently more stable (i.e., compare formulation D, Fig. 6 vs Fig. 7). Clearly, the fact that the excipient system is amorphous is not a sufficient condition for stability enhancement. It seems likely that dextran 40 destabilizes the native conformation of hGH in the solid state, thereby leading to increased aggregation. Since our assay methodology does not distinguish between covalent and noncovalent aggregates, the question of whether or not aggregation in the dextran 40 formulation is structurally identical to aggregation in the other formulations remains unresolved.

One may use the data in Fig. 6 to evaluate the temperature dependence of both chemical decomposition and aggregation, although the presence of two parallel chemical reactions (deamidation and oxidation) and the lack of a defined rate constant for aggregation limit the mechanistic interpretation of such results. The mean effective "activation energy" for reverse-phase decomposition of formulations A–D is 17.1 ± 0.6 kcal/mol, with no significant differences between the formulations. The uncertainty given is simply the standard error of the mean. Note that this "activation energy" is roughly a mean of the activation energies for methionine oxidation and asparagine deamidation (Fig. 4). Using the ratio of extent of aggregation increase during storage divided by the time of storage as a quasi rate constant for aggregation, the activation energies for aggregation are as follows: A, 24 kcal/mol; B, 32 kcal/mol; C, 27 kcal/mol; and D, 31 kcal/mol. While the differences among formulations B, C, and D are small and probably not significant, the "activation energy" for aggregation in formulation A appears to be slightly lower than for formulations B and D, a result consistent with the stabilizing effect of these "lyoprotectants."

Effect of Level of Phosphate Buffer. The effect of the level of buffer ($Na_2HPO_4 \cdot 7H_2O$) on both the chemical stability of formulation D (glycine:mannitol) and the aggregation in formulation A (no excipients) is summarized in Fig. 8. Chemical decomposition appears to be minimized at buffer levels of 0.227 and 0.454 mg buffer/mg hGH and is maximized at the "0.114" level of buffer. Aggregation appears to be at a maximum at the "0.227" level of buffer. No systematic trends were noted with either aggregation in excipient system D or chemical decomposition in system A (data not shown).

Phosphate is reported to be a catalyst for deamidation (24), and aggregation in solution is sensitive to the concentration of phosphate buffer (minimum aggregation at 5 m*M* buffer) (24). Further, crystallization of sodium phosphate salts during freezing can cause massive pH decreases during the freezing process (8). Crystallization would be more probable at higher levels of buffer in systems without lyoprotectants. Thus, one might expect variations in stability as a function of buffer level. It is possible that a combination of the above effects is responsible for the trends (and lack of trends) found in our studies. Phenomenologically, although the magnitude of the buffer effects is small, the level of phosphate buffer does appear to be a relevant formulation variable in the stability of freeze-dried hGH.

Effect of pH. Deamidation of asparagine residues in aqueous lysozyme increases with an increase in pH (25), and the recent summary of hGH results in solution (24) arrives at the same generalization. While pH has no meaning in the freeze-dried solid, the solution pH before freeze-drying obviously determines the extent of ionization of the protein both in solution and in the solid.

The effect of starting solution pH on the chemical stability of freeze-dried hGH is summarized by Fig. 9. In this series, the reverse-phase decomposition did not appear fully consistent with first-order kinetics, although whether this observation is a result of a "real" effect or the result of a larger than expected assay error is unknown. However, to avoid the possibility of distorting the data by forcing a first-order fit, chemical decomposition data are reported as loss

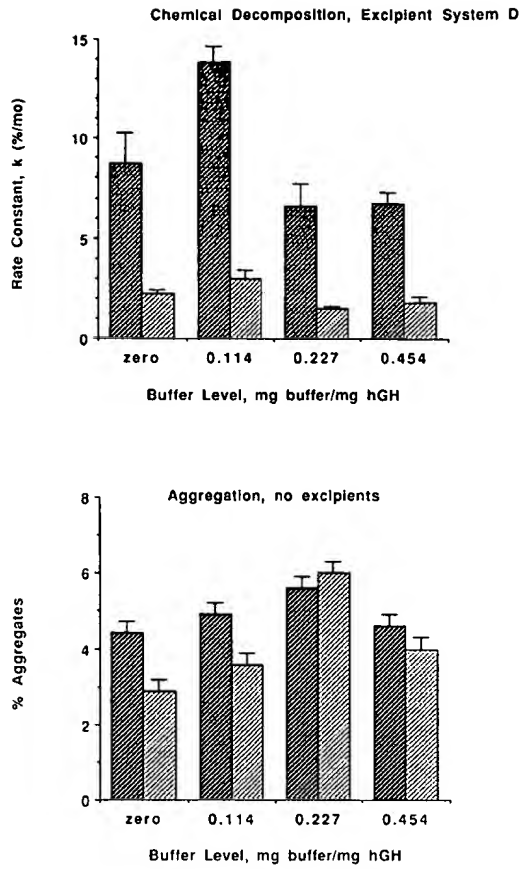

Fig. 8. The effect of sodium phosphate buffer level on the stability of freeze-dried hGH. Buffer level is in mg $Na_2HPO_4 \cdot 7H_2O$/mg hGH with pH 7.4. Chemical decomposition: dark shading, 40°C; light shading, 25°C. Aggregation: dark shading, 3 weeks at 40°C; light shading, 2 months at 25°C.

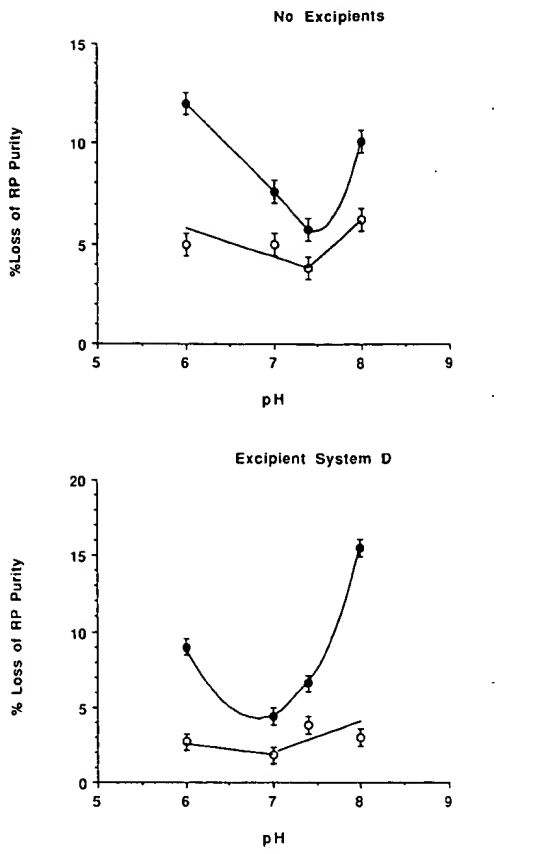

Fig. 9. The effect of pH on loss of reverse-phase purity for freeze-dried hGH. The samples at pH 6, 7, and 8 do not contain buffer. These solutions were adjusted to the appropriate pH by titration with H_3PO_4 or NaOH. Filled circles, increase in decomposition after 3 weeks at 40°C; open circles, increase in decomposition after 2 months at 25°C.

of reverse-phase purity at fixed time points. Three weeks at 40°C is the mean of 2 and 4 weeks at 40°C, and 2 months at 25°C is the mean of 1 and 3 months. Error bars represent estimates of the standard error. The data at 40°C show a clear minimum in chemical decomposition in the pH range 7–7.5. Data at 25°C are consistent with a minimum in decomposition in the same pH range, but here, the observed variation with pH is minimal. The increase in reactivity at 40°C as the pH increases is expected based on previous data in aqueous solution (24,25), but the increase in reactivity at pH 6 appears to be unique to the solid state. The lack of a strong pH effect at 25°C coupled with the significant pH effect at 40°C implies that the effective activation energy also passes through a minimum around pH 7, a curious observation for which we have no explanation.

Aggregation in pure hGH (no excipients) at 40°C is slightly greater at pH 6 than at the other pH's studied, but aggregation at 25°C is independent of pH (Fig. 10). With excipient system D (glycine:mannitol), aggregation appears to decrease slightly as the pH increases from 6 to 7.4. At pH 8, aggregation at 40°C increases dramatically (no data were obtained at pH 8 and 25°C). These results are in sharp contrast to aggregation in hGH solutions where formation of insoluble aggregates decreases with increasing pH over the pH range 6–8 (24).

Effect of Sodium Chloride Level. Since sodium chloride is often included in parenteral formulations as a tonicity modifier, the effects of sodium chloride levels equivalent to "normal" saline (0.9% NaCl) and dilute saline (0.1%) were investigated. Even at the lower level of saline, hGH samples freeze-dried with sodium chloride do not reconstitute to form a clear solution. Some of the hGH remains in the form of insoluble aggregates, producing a turbid solution. Turbidity increases as the level of NaCl increases, and at a given level of NaCl, turbidity is greater for formulation A than for formulation D. While quantitative size-exclusion HPLC data could not be obtained, the samples were completely soluble in the denaturing mobile phase used for the reverse-phase HPLC assay, and reverse-phase purities of the initial freeze-dried samples were essentially the same as found for samples prepared in the absence of sodium chloride. These observations indicate noncovalent aggregation.

A comparison was made of visual turbidity between solutions frozen (and then thawed) and the corresponding solutions freeze-dried (and then reconstituted with water). The freeze-thawed solutions were all essentially clear, whereas

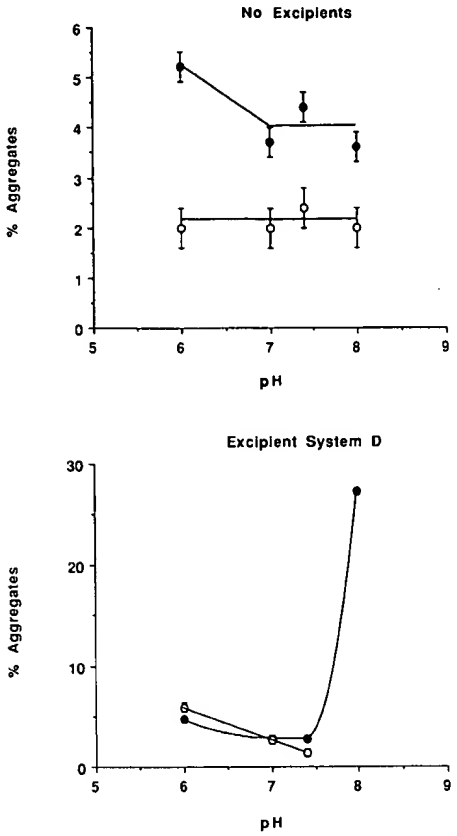

Fig. 10. The effect of pH on aggregate formation in freeze-dried hGH. The samples at pH 6, 7, and 8 do not contain buffer. Filled circles, aggregation after 3 weeks at 40°C; open circles, aggregation after 2 months at 25°C.

the freeze-dried–reconstituted solutions containing NaCl were turbid, as indicated above. The NaCl-induced conformational changes leading to precipitation appear to occur during drying and not during freezing.

Chemical stability of the freeze-dried solid is also decreased by the presence of NaCl (Fig. 11). Rate constants for loss of reverse-phase purity increase in nonlinear fashion with increasing levels of sodium chloride. Limited data for samples stored at 25°C (1 month only) show qualitatively the same trend (data not shown) as the 40°C results. At each level of NaCl studied, the stability of formulation D (glycine: mannitol excipients) is superior to formulation A (no lyoprotectants), indicating that the glycine:mannitol excipient system moderates, to some extent, the detrimental effect of NaCl.

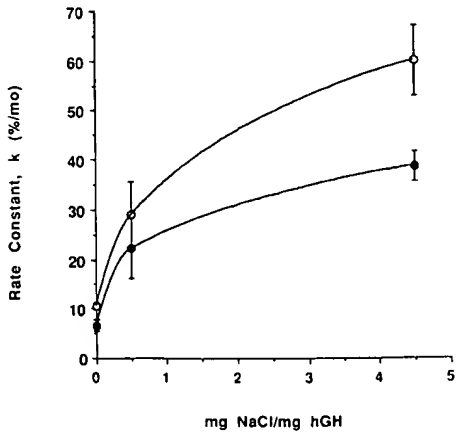

Fig. 11. The effect of NaCl on reverse-phase decomposition of freeze-dried hGH at 40°C. Open circles, formulation A; filled circles, formulation D.

In principle, the effect of NaCl on the stability of freeze-dried hGH could be attributed to either an "ionic strength" effect or a specific ion effect (Na^+ or Cl^-). The available data suggest that the detrimental effect of saline is due primarily to Cl^-. In the systems studied without added sodium chloride, the concentration of Na^+ contributed by the buffer varied from 0 to 7 m*M*, and the ionic strength of electrolyte (i.e., buffer) varied from 0 to 10 m*M* (Fig. 8). Neither aggregation nor chemical stability was adversely affected by the higher levels of sodium phosphate. The total sodium ion concentration and ionic strength for the 0.1% saline solution (with 0.227 mg buffer/mg hGH) are 20 and 22 m*M*, respectively. As discussed, freeze-drying from this solution caused significant aggregation and turbidity. Thus, either an enormous discontinuity exists in aggregation phenomena as a function of $[Na^+]$ and/or ionic strength or the aggregation phenomena in samples freeze-dried from saline are due to the presence of Cl^-. We believe the latter is more plausible.

SUMMARY AND CONCLUSIONS

While irreversible changes in hGH during the freeze-drying process are minimal, chemical decomposition via methionine oxidation and asparagine deamidation and aggregation do occur on storage of the freeze-dried solid, and such irreversible changes are sensitive to the choice of formulation. Even in a nominal nitrogen headspace (≈0.5% O_2), decomposition via methionine oxidation is significant. A combination of mannitol and glycine provides the greatest protection against degradation, particularly for degradation via aggregation. While it is postulated that an excipient system that remains at least partially amorphous is necessary for stabilization, an amorphous excipient system is clearly not a sufficient condition for stability, as dextran 40 formulations show poor stability toward aggregation. Stability is greatest in the pH range 7–7.5, with severe aggregation being observed at high pH. The level of sodium phosphate buffer does impact on stability, although stability is not a monotonic function of buffer concentration. Freeze-drying in the presence of NaCl produces severe aggregation and precipitation during the drying stage of freeze-drying.

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